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*TITLE: Annexin II-Mediated Ca++ Influx Regulates Endothelial Cell (EC) Apoptosis and Tumor Angiogenesis

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14. ABSTRACT

Angiostatin (AS), the first four kringle domain (K1-4) of plasminogen (PLG), blocks angiogenesis and breast cancer progression almost 95 % in xenograft model. Despite great therapeutic potential in breast cancer its mechanism of action is unclear. We previously reported that AS ligand binds to endothelial cell surface annexin II and blocks PLG bindings. Emerging role of annexin II in cancer prompted us to investigate its possible mechanism in breast cancer. In this study we report that that annexin II gene and protein abundantly expressed in highly invasive and metastatic breast cancer cells MDA-MB231 but not in non invasive MCF-7 cells. Annexin II expression is regulated by proangiogenic growth factors. Growth factors also phophorylate tyrosine residue of annexin II in MDA-MB231 cells indicating the involvement of signal transduction mechanism. MDA-MB231 cells activated PLG to plasmin (PL) in time dependent manner whereas MCF-7 cells lacking annexin II expression failed to activate PLG indicating that MDA-MB231 cells require annexin II for PLG activation and may be involved in invasion and migration. Our data indicates that MDA-MB231 cells induced invasion through ECM in PLG dependent manner but MCF-7 failed to invade and migrate suggesting specific role of annexin II mediated PL generation in invasion and migration. It is possible to block breast cancer invasion and migration by blocking annexin II and may be an attractive target.

15. SUBJECT TERMS
Annexin II, angiogenesis, breast cancer, invasion

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Introduction: It has been recognized for decades that growth and development of breast cancer is dependent on angiogenesis [1]. Weidener *et al* reported that microvessel density (either count or grade serves as a measure of tumor angiogenesis) in invasive breast carcinoma is associated with metastasis and, thus, may be a prognostic indicator [2, 3]. Increase in tumor microvasculature not only allows for rapid growth of tumors but may also provide the means for tumor cells to enter and exit the circulation during hematogenous tumor spread. In addition, endothelial cells (EC) may play a significant role in tumor progression by providing invading tumor cells with essential molecules necessary for extracellular matrix (ECM) degradation such as proteolytic enzymes [4]. Therefore, tumor angiogenesis plays an active and critical role in tumor progression and metastasis.

Regulation of angiogenesis is a fundamental mechanism to control of tumor progression [1]. Using this novel approach, Folkman and colleagues identified angiostatin (AS), an internal fragment of plasminogen (PLG) spanning kringle 1-4 region, as one of the most powerful angiogenesis inhibitors [5]. These investigators further demonstrated 95% regression of human breast cancer by AS treatment in xenograft mice model without toxicity [6]. Later other investigators also demonstrated impressive anti-human breast cancer activity by AS gene therapy [7]. Recently, AS therapy has been reported to inhibit breast cancer induced bone metastasis [8]. Angiostatin was the first anti-angiogenic protein to enter therapeutic cancer clinical trials.

Despite the potential anti-breast cancer therapeutic value of AS, its clinical utility is hampered by limited availability of the recombinant bioactive AS. Human pharmacokinetics, particularly a short half-life in circulation [6] makes drug delivery challenging. Since discovery of AS, various mechanism(s) of action for AS have been suggested, including from our laboratory. Despite the identification of multiple receptors for AS [9-11], current knowledge of how AS inhibits breast cancer growth and metastasis is still unclear. If mechanism of action for AS is identified, more candidate drugs can be developed to target receptor(s).

To delineate the AS's molecular mechanism we identified, purified and characterized a potential receptor for AS from EC surface [12, 13]. Using proteomics approach we have identified this protein as annexin II [12] and proposed a likely mechanism in angiogenesis. To explore its mechanism in breast cancer we have identified expression of AS's receptor annexin II in invasive human breast cancer cell line (MDA-MB231) in vitro and human ductal carcinoma in vivo [14]. Annexin II is one of the most abundant EC surface fibrinolytic receptors for PLG [15]. It is capable of converting inactive enzyme PLG to highly active protease plasmin (PL) almost 300 fold [16]. Consistent with previous observations we found that invasive MDA-MB231 cells expressing high levels of annexin II were also capable of converting PLG to PL with high efficiency. This is in contrast to poorly invasive cell line (MCF-7), which failed to convert PLG to PL (see preliminary data). Pericellular plasmin-mediated degradation of extracellular matrix (ECM) has been reported to induce tumor cell invasion, metastasis and tumor progression [17-19]. In addition, plasmin librates matrix bound pro growth factors bFGF and VEGF during proteolysis of ECM [20]. Furthermore, plasmin mediated proteolytic processing of growth factors is required to induce cell proliferation and tumor progression [21]. Thus, annexin II may play a pivotal role in the **pro-anti-angiogenic switch mechanism** through precise regulation of PLG and growth factors activation. Recent studies on PLG knockout mice (PLG-/-) reported an absolute requirement for plasmin in cancer invasion, angiogenesis and tumor progression [22-24]. This suggests that invasive breast cancer cells generate plasmin with a prominent role in ECM degradation, invasion for tumor progression and metastasis to distant sites. In this context, plasmin inhibitors have been tested in clinical setting as well as in xenograft mouse model of

cancer and showed promising results [25-27]. In our laboratory we have made a direct attempt to block in vivo annexin II mediated plasmin generation in mouse model of Lewis Lung Carcinoma (LLC) and found remarkable inhibition of tumor growth by monoclonal antibody mediated blocking of annexin II [28] (see appendix).

Bone is very common metastatic site for breast cancer. Emerging studies suggest that AS treatment inhibits MDA-MB231 induced bone metastasis through direct anti-osteoclastic activity [8]. It is interesting to note that annexin II increases osteoclast formation and bone resorbtion [29, 30]. These reports further support the link between annexin II and breast cancer metastasis and also strongly support our findings. It is likely that AS inhibiting breast cancer progression and metastasis by blocking annexin II functions in invasive breast cancer as we propose. Targeting this component of fibrinolytic system (PLG/PL) has yielded exciting results in the war against cancer [31, 32]. It remains to be seen whether targeting fibrinolytic receptor annexin II will have clinical efficacy yet to be answered.

Another central function of annexin II in the cell is its role in signal transduction mechanism. Annexin II is a calcium and phospholipid binding protein and major in vivo substrate for protein tyrosine kinase and PKC [33, 34]. It binds to the cytoskeleton protein actin and helps to organize into dynamic meshwork of actin fibers. Recent reports suggest that AS treatment induces the rise in intracellular calcium ($[Ca^{++}]_i$) through the PI-3 kinase signaling pathways [35], which requires reorganization of the actin cytoskeleton. Reports suggest that contact between breast cancer cells and EC induces an immediate and transient increase in intracellular $[Ca^{2+}]$ [36] indicating that signal transduction pathways are involved in these interactions. It is conceivable that targeted disruption of annexin II by AS treatment may disorganize actin microfilament architecture, affecting cellular physiology such as cell-cell interaction, migration and proliferation [37]. Targeted disruption of actin microfilament assembly has been demonstrated in invasive (MDA-MB231) breast cancer cell death and morphological changes in cell shape [38]. Annexin II has a limited tissue distribution and is not typically expressed in normal and mature organs such as liver and brain (Sharma et al, unpublished observations). However, its expression in liver cancer and brain tumor are highly up regulated [28, 39-41]. We found that quiescent EC do not express annexin II but exposure to growth factors up-regulates annexin II expression suggesting its possible role in cell proliferation, angiogenesis and tumor progression. Recently we reported that anti-annexin II antibody perturbs cell growth and induces EC cell apoptosis in a dose dependent manner; disrupting blood vessel formation in vitro [42]. These data suggest that disruption of the cell surface exposed annexin II may play a pivotal role in signal transduction mechanism.

One of the attributes that metastatic cells must develop is the ability to degrade the ECM in order to initiate tumor progression and induce metastatic spread. To accomplish this, metastatic cells may activate annexin II fibrinolytic activity to generate plasmin, which in turn cleaves basement membrane constituents to clear the path for cellular invasion and migration. This is one of the prerequisite steps of angiogenic and metastatic processes. It is likely that AS binding to annexin II acts as antagonist and may disable the plasmin generation capacity of the cell and potentially inhibits invasion [22] cell migration [43] and proliferation [44, 45].

Our novel studies in breast cancer indicate that annexin II mediated plasmin provides a model system with which to further probe the molecular mechanism underlying breast tumor progression. Annexin II protein expression appears to act as a tumor and metastasis promoter by cell surface mediated plasmin generation. Our preliminary data suggest that invasive breast cancer cells are equipped with the machinery necessary for degradation of ECM initiating

angiogenesis and metastasis. It is not unreasonable to mention that AS mediated remarkable inhibition of breast cancer and metastasis [8] seen by Folkman and colleagues [6] may be due to blocking annexin II and its mediated signaling. Results obtained from this study will establish the role of fibrinolytic receptor annexin II in angiogenesis, metastasis and breast tumor progression and may lead to design of effective breast cancer therapeutics.

Body: We have successfully completed the task 1 as described in statement of work. We had some technical difficulty to perform the task 2. Now we have identified and solved the technical problems of the experiments in task 2. We are granted no cost extension for one year to complete the experiments described in task 2. The results of task 1 are summarized below.

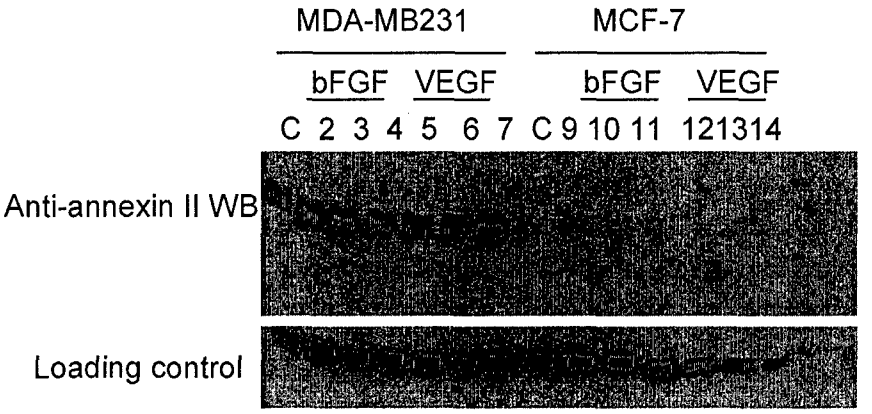
Task 1: growth factors-mediated tyrosine phosphorylation and temporal expression of annexin II in EC, MCF-7 and MDA-MB231 cells.

a) Effect of angiogenic growth factors on annexin II expression.
b) Determine the growth factors stimulated tyrosine phosphorylation in EC, MCF-7 and MDA-MB231 cells.

Results:

Annexin II expression in invasive and non-invasive breast cancer cells and its regulation by growth factors. Our experimental data suggest that annexin II is specifically expressed in invasive breast cancer cells but not in hormone receptor positive non invasive breast cancer cells MCF-7.

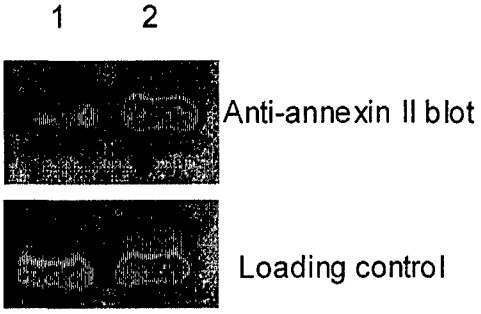
Fig.1



Temporal regulation of annexin II expression in invasive and non-invasive breast cancer cells by angiogenic growth factors VEGF and bFGF: Invasive (MDA-MB231) and noninvasive breast cancer (MCF-7) cells were cultured in presence of bFGF and VEGF for various time points. Cells were lysed and 10 μg protein was analyzed for annexin II expression by Westren blot. Lane C Control (no treatment); Lanes 2,3,4 treated for 6 ,4 and 2 hours respectively. Lanes 5,6,7 were treated for 6 ,4 and 2 hours respectively. Lanes 9,10 and 11 were treated for 6 ,4 and 2 hours respectively and 12,13 and 14 were for 6 ,4 and 2 hours respectively.

Annexin II expression is up regulated by bFGF after 2 hours in invasive breast cancer cells MDA-MB231. Our results indicate that bFGF up regulates annexin II expression in MDA-MB231 cells within 2 hours of treatment.

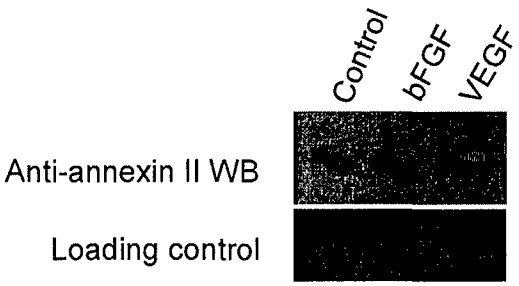
Fig.2



Over expression of annexin II by bFGF: bFGF was incubated in serum free medium for 2 hours. Cell lysates were analyzed for annexin II expression. Lane #1 control; lane # 2 treated with bFGF

Angiogenic cytokines induce annexin II expression in bovine aortic endothelial cells (BAEC). Our experimental evidence indicates that annexin II expression is up regulated by angiogenic cytokines bFGF and VEGF suggesting its possible role in angiogenesis.

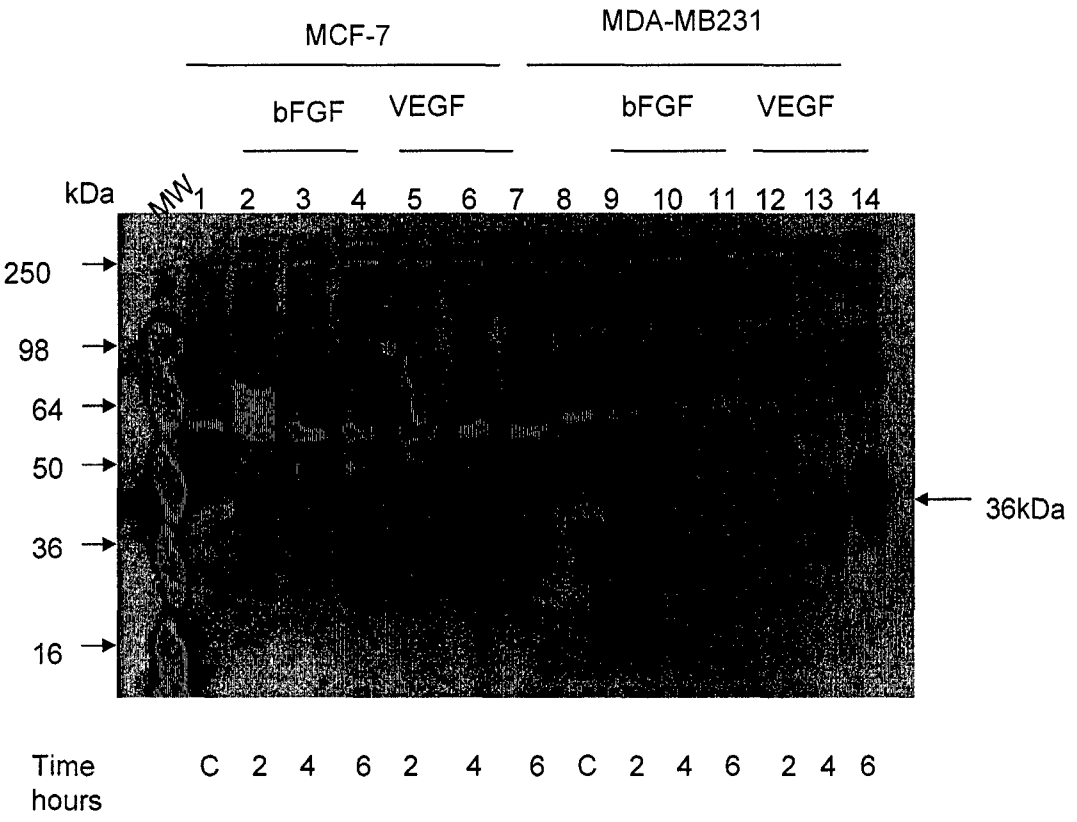
Fig.3



Growth factors induce annexin II expression in endothelial cells: Both bFGF and VEGF were incubated in serum free cultured medium for 2 hours. Cell lysates were prepared and 10-μg protein was analyzed for annexin II expression

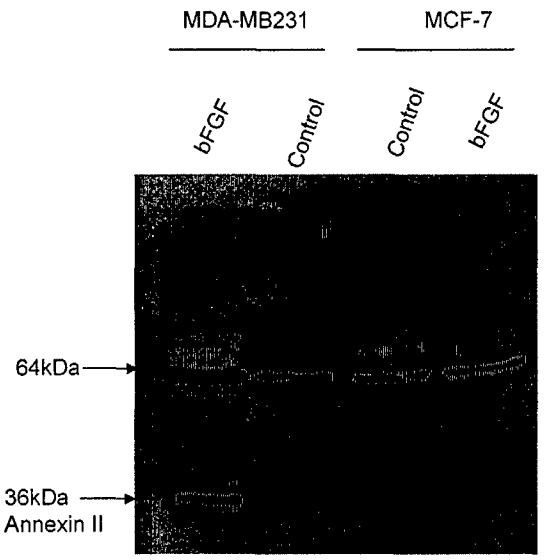
Effect of growth factors on tyrosine phosphorylation of proteins in MCF-7 and MDA-MB231 cells: Cells were stimulated by growth factors for various times to analyze the tyrosine phosphorylation of proteins. Cell lysates were separated by SDS-PAGE, transferred on to membrane and probed with anti-Tyr-P monoclonal antibody. Data presented in figure 4 indicates that both growth factors (bFGF and VEGF) induce extensive and distinct tyrosine phosphorylation of proteins in MCF-7 and MDA-MB231 cells. Importantly, we have observed specific tyrosine phosphorylation of 36-kDa protein in MDA-MB231 cells but not in MCF-7 cells. This protein showed same electrophoretic mobility as annexin II. Immunoprecipitation analysis confirmed that 36-kDa phosphoprotein is annexin II **(Fig.4 B).**

Fig.4 A



bFGF induces tyrosine phosphorylation of annexin II specifically in invasive breast cancer MDA-MB231 cells: Cells were stimulated by bFGF for 2 hours in serum free medium. After the treatment cells were lysed and immunoprecipitated by anti-Tyr-p monoclonal antibody and probed with anti-annexin II antibody.

Fig.4 B



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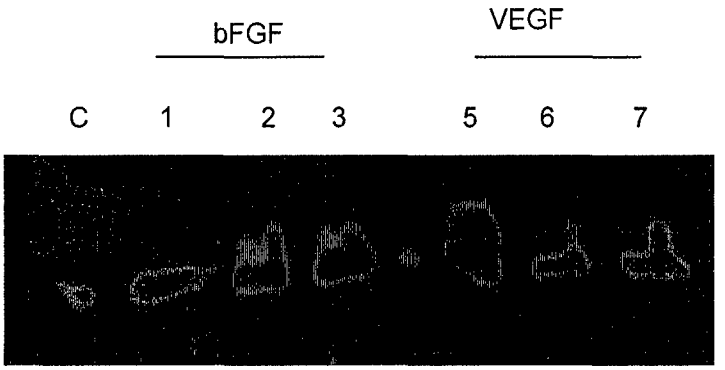
Effect of growth factors on tyrosine phosphorylation of proteins in BAEC: In contrast tobreast cancer cells we have observed time dependent phosphorylation of 64kDa protein in BAEC. We did not observe the phosphorylation of annexin II in BAEC.

Fig.5.



Effect of growth factors on tyrosine phosphorylation in EC: Bovine aortic endothelial cells (BAEC) were stimulated with growth factors for various time points as indicated in figure. Phosphotyrosine reactive proteins were identified using monoclonal antibodies Tyr-P.

Task 2: The role of annexin II mediated Ca^{++} signaling in cell survival and apoptosis

a) Perform assays to determine intracellular **Ca^{++}**
b) Apoptosis assays after blocking anti-annexin II antibody

Results:

Intracellular Ca^{++} levels after angiostatin (AS) treatment in bovine aortic endothelial cells (BAEC): We have found that AS (5 mg/ml) elicited transient, approximately threefold increases in intracellular **Ca^{++}** concentration ([**Ca^{++}**]i). When we added EGTA in the medium (specific chelator of **Ca^{++}**) which abolished AS induced ([**Ca^{++}**]i). These data may suggest that intracellular **Ca^{++}** signaling may initiate or mediate some of the cellular actions of angiostatin in BAEC. Interestingly, exposure of AS to breast cancer cells (MCF-7 and MDA-MB231) did not elicit any effect on intracellular **Ca^{++}** levels. These data suggest that AS do not affect cancer cells directly rather it induces endothelial cell apoptosis.

Fig.6.

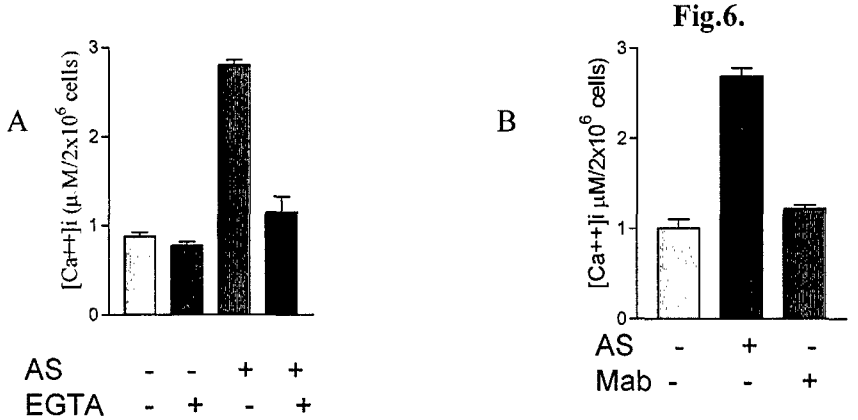
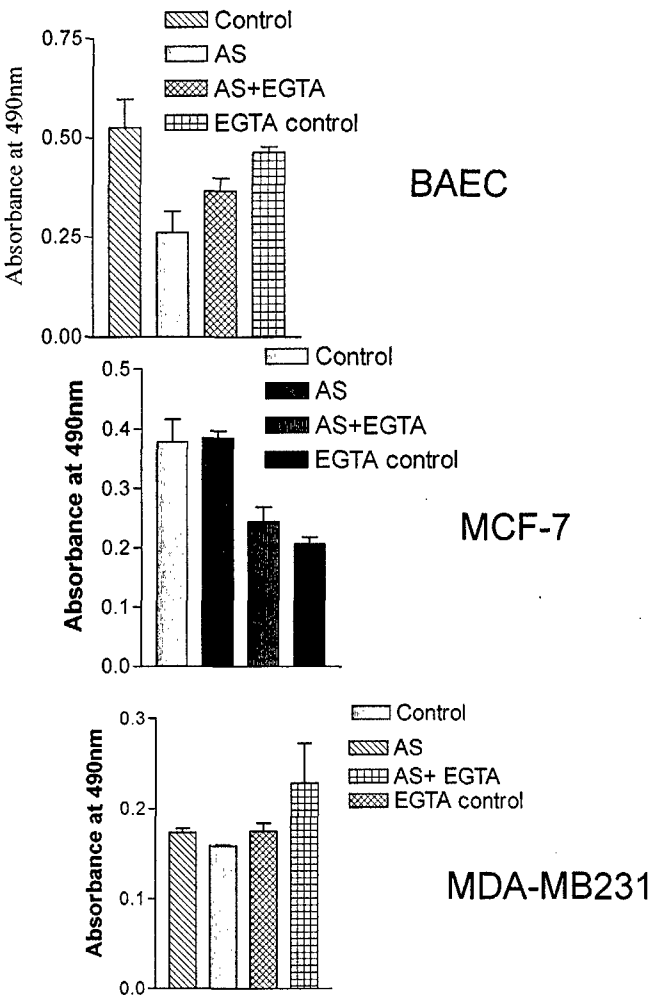


Fig.6. Cytosolic Ca^{++} concentration of untreated, AS and AS/EGTA treated BAE cells. BAE cells were cultured in the presence and absence of AS, EGTA or AS and EGTA for 1 day. The cytosolic Ca^{++} concentration was measured using Fura -2AM. Florescence was measured using flurorimeter using excitation wavelength of 340 nm (Ca^{++} bound form of Fura-2AM) and emission wavelength of 510 nm. The fluorescence maximum (Fmax)was determined by addition of 2 pM ionomycin (Calbiochem-Novabiochem), and the fluorescence minimum (Fmin) was determined in the presence of 1 mM EGTA/10 mM Tris. $[Ca^{++}]i$ was calculated according to the following equation: $([Ca^{++}]i) = Kd \times [(F - Fmin)/(Fmax - F)]$ with *Kd* 224nM.

AS triggers Ca^{++}-dependent BAEC cell death: Our previous data suggest that AS treatment increases intracellular Ca^{++} in BAE cells. Surprisingly, we did not observed any effect on breast cancer cell intercellular calcium levels. Calcium believed to be a key factor in apoptosis. Thus, we tested the role of intracellular Ca^{++} in cell death. We chelated Ca^{++} from culture medium by EGTA (0.5 mM for 2 days) and found that AS-triggered (10 μg/ml for 2 days) cell death was significantly prevented (Figs. 1*A* and 2*A*), although there were slight decreases of cell numbers in EGTA-only groups. Cell viability was measured using Promega kit as we reported previously (46). Although AS treatment has been shown to inhibit breast cancer growth about 90% in xenograft model but in our experimental model AS treatment showed no effect on breast cancer cell lines in vitro(MCF-7 and MDA-MB231 cells) (Figs. B and C).

Fig.7.

AS triggers Ca^{++} -dependent BAEC cell death: BAEC were treated as follows: control, AS (10μg/ml), AS+EGTA and EGTA (0.5mM) for 2days. Cell viability was measured using Promega's kit as we reported earlier. Absorbance was measured at 490nm.



Effect of AS and anti-annexin II antibody (Mab) on BAEC viability. Studies have demonstrated that AS *in vitro* inhibits endothelial cell proliferation, migration, tube formation and induces apoptosis. Most of the studies available in the literature were focused to evaluate the clinical utility of angiostatin to regress the tumor growth in vivo. Despite the identification of multiple receptors for angiostatin, the question still remains to be answered as to how angiostatin inhibits endothelial cell proliferation and induces apoptosis? Is there any down-stream cellular signaling mechanism initiated by angiostatin or is AS simply cytotoxic to endothelial cells? Since AS binds to annexin II we determined if blocking of annexin II with a high affinity anti-annexin II monoclonal antibody induces cell death similar to AS. BAE cells were incubated with various concentrations of anti-annexin II antibody for 72 hours. In a parallel wells AS was used as positive control and same concentration of mouse IgG was used as negative control. Surprisingly, monoclonal antibody to annexin II reduced cell viability in dose dependent manner. Photomicrograph showed cell death induced by antibody (Fig.8). It is possible that AS interaction with annexin II may alter Ca^{++} homeostasis, which triggers the whole cascade of terminal differentiation events, including apoptosis.

Fig.8.

B

A

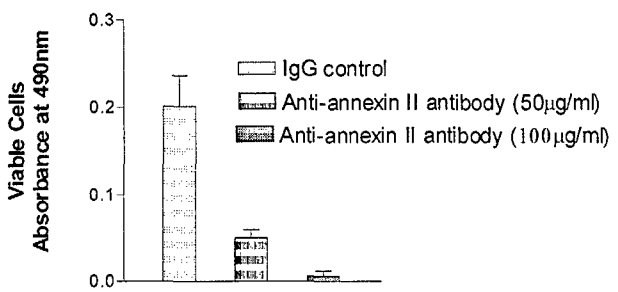


Fig.8. Effect of anti-annexin II antibody on BAE cell viability: BAE cells were seeded in 96 wells plates. Various concentrations of anti-annexin II antibody were incubated in medium after the cells stimulated with bFGF. BAE cells viability was determined by Promega's kit (A). Cells treated with equal amount of mouse IgG were considered control. Cells treated with angiostatin considered positive control. Phase contrast photomicrograph showing the morphology of the cells after treatments (B).

Key Achievements:

1. Calcium binding protein annexin II specifically express in invasive breast cancer cells (MDA-MB231) but not in hormone receptor positive non-invasive breast cancer cells (MCF-7).
2. Growth factors up regulate annexin II expression in MDA-MB231 cells.
3. Annexin II acts as receptor for plasminogen on invasive MDA-MB231 cells and facilitates activation of plasminogen to plasmin. In sharp contrast, MCF-7 cells lacking annexin expression failed to activate plasmin.
4. MDA-MB231 cells were able to degrade extracellular matrix (ECM) in plasminogen dependent manner but MCF-7 cells failed to invade and migrate through ECM suggesting that annexin II mediated plasminogen activation is required for cellular invasion, migration and angiogenesis.
5. Phosphorylation of annexin II is observed only in MDA MB231 cells but not in MCF-7 cells.
6. Angiostatin (AS), a powerful inhibitor of breast cancer growth, does not induce breast cancer cell apoptosis. AS specifically induces endothelial cell apoptosis possibly through Ca^{++} signaling via its interaction through annexin II.

Reportable Outcomes: The outcome of this study was presented in 4th Era of hope meeting in Philadelphia sponsored by the DOD ***(Proceedings of Era of Hope P10-14, page 77 2005)***

Conclusions: On the basis of our experimental evidence we concludes that specific expression of annexin II on invasive breast cancer cells MDA-MB231 regulates plasminogen activation to plasmin which in turn degrades ECM and facilitates cellular invasion, migration and angiogenesis dependent tumor progression. Angiogenic cytokines induce BAEC proliferation and correlates with annexin II expression suggesting possible role in cell proliferation and angiogenesis.

AS treatment directly targets endothelial cells and induces apoptosis not the breast cancer cells. This endothelial cell apoptosis is may be through Ca^{++} signaling mechanism.

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